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Gene Therapy for Vascular Smooth Muscle Cell Proliferation After Arterial Injury

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Accumulation of vascular smooth muscle cells as a consequence of arterial injury is a major feature of vascular proliferative disorders. Molecular approaches to the inhibition of smooth muscle cell proliferation in these settings could potentially limit intimal expansion. This problem was approached by introducing adenoviral vectors encoding the herpesvirus thymidine kinase (tk) into porcine arteries that had been injured by a balloon on a catheter. These smooth muscle cells were shown to be infectable with adenoviral vectors, and introduction of the tk gene rendered them sensitive to the nucleoside analog ganciclovir. When this vector was introduced into porcine arteries immediately after a balloon injury, intimal hyperplasia decreased after a course of ganciclovir treatment. No major local or systemic toxicities were observed. These data suggest that transient expression of an enzyme that catalyzes the formation of a cytotoxic drug locally may limit smooth muscle cell proliferation in response to balloon injury.

Injury of the arterial wall induces the synthesis of gene products that stimulate smooth muscle cell migration and proliferation, leading to intimal hyperplasia (*1*). This process contributes to the pathogenesis of several cardiovascular disorders, including atherosclerosis. Another common and clinically significant setting for such injury is balloon angioplasty. In this procedure, a stenotic artery is dilated mechanically with a balloon on a catheter to restore blood flow in coronary arteries. However, in many cases, a reactive cellular proliferative response leads to regrowth of cells locally that impinges on the lumen and compromises blood flow. This process, called restenosis, has been refractory to conventional treatment approaches such as antiplatelet agents, angiotensin-converting enzyme antagonists, or cytotoxic drugs in humans (*2*, *3*). Genetic interventions to limit cellular proliferation at specific sites in the arterial wall could thus provide insight into the pathogenesis and possible treatments of vascular proliferative diseases.

Balloon injury of porcine arteries provides an animal model relevant to human vascular diseases. The arterial bed of swine has a size and structure that is histologically and biochemically similar to human coronary arteries, including a developed intima consisting of elastic tissue, collagen, scattered smooth muscle cells, and endothelium (*4*). Swine are also susceptible to spontaneous (*5*) and diet-induced atherosclerosis (*6*) that morphologically and topographically resembles human atherosclerosis (*7*). In addition, several inherited genetic cardiovascular disorders, including hyperlipidemia and deficiency of von Willebrand's factor (vWF), share similar features to the corresponding human disease (*5*, *8*). Balloon angioplasty of porcine arteries results in smooth muscle cell proliferation and eccentric intimal thickening (*9*–*11*), and the histological appearance is very similar to the proliferative intimal tissue of human restenosis (*11*, *12*). Direct gene transfer into porcine arteries has also been achieved (*13*), and the biologic effects of several recombinant genes have been analyzed in genetically modified arteries (*14*).

We reasoned that local delivery of an antiproliferative agent during the peak of smooth muscle cell division after balloon injury might limit intimal hyperplasia. Previous failures to accomplish this effect with cytotoxic drugs were likely due to rapid removal of the drugs by the arterial circulation. One common approach to the selective elimination of dividing cells is to express a recombinant gene, herpesvirus thymidine kinase (*tk*), which converts the nucleoside analog ganciclovir into an active toxic form in transduced cells (*15*, *16*). The tk enzyme phosphorylates ganciclovir in vivo, and its subsequent incorporation into DNA induces chain termination in dividing cells, causing cell death (*17*). The introduction of this recombinant gene into normal arteries has no effect on nondividing cells (*18*). To develop genetic interventions for balloon injury in the porcine model, we defined the kinetics of smooth muscle cell proliferation in the intima after balloon injury to the iliofemoral artery. Proliferation was observed 24 hours after injury as determined by immunohistochemical staining of serial arterial sections with a monoclonal antibody to 5-bromo-2'-deoxycytosine (BrdC) to detect its incorporation into cellular DNA and an antibody to smooth muscle α-actin to identify smooth muscle cells (*19*). Incorporation of BrdC was maximal at 4 to 7 days after the injury and subsided by 14 days (Fig. 1). Continued expansion of the arterial intima occurred preferentially by deposition of extracellular matrix through 21 days (*20*).

The efficacy of the herpesvirus tk in limiting porcine vascular smooth muscle cell growth was first assessed after gene transduction and exposure to ganciclovir in vitro. Cells infected with a control adenoviral vector lacking E1 and containing no insert (ADV-ΔE1) were resistant to ganciclovir at high concentrations. In contrast, cells infected with an adenoviral vector expressing tk (ADV-tk) were completely nonviable within 48 hours after exposure to ganciclovir (Fig. 2A). Mixtures of transduced and nontransduced porcine vascular smooth muscle cells showed that as few as 10% of cells transduced with this gene conferred susceptibility of the entire culture to ganciclovir. Thus, the bystander effect, previously demonstrated in a variety of malignancies (*16*, *18*), effectively inhibited vascular smooth muscle cells in vitro.

Although several studies have used retroviral vectors to deliver a recombinant *tk*

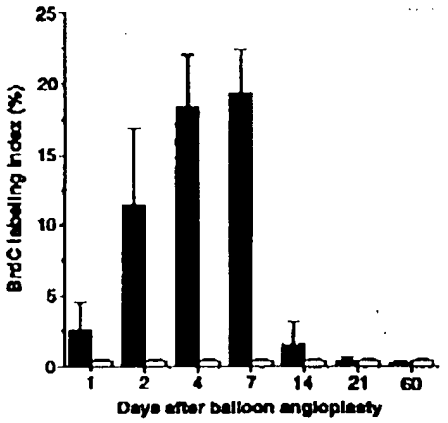

Fig. 1. Effect of balloon injury on smooth muscle cell proliferation in injured porcine arteries. Proliferation of intimal cells was measured in injured porcine iliofemoral arteries with BrdC labeling 1 to 60 days after balloon angioplasty (n = four arteries at each time point, two arteries per animal). Intimal cell proliferation was measured by counting the number of labeled and unlabeled nuclei in four quadrant cross sections of balloon-injured and uninjured arteries with a microscope-based video image analysis system (*14*, *23*). Injured iliofemoral arteries (solid bars) and uninjured carotid arteries (open bars) were examined in the same animal. Standard error bars are shown. Additional immunohistochemical studies with an antibody to smooth muscle α-actin identified proliferating intimal cells as smooth muscle in origin.

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gene to selected tissues (*16, 18*), the relative percentage of cells transduced in vivo can be limiting. Studies have suggested that the efficiency of gene transfer into arteries can be improved with adenoviral vectors (*21*). To determine whether expression could be achieved at the appropriate sites in vivo, we introduced adenoviral vectors into injured porcine iliofemoral arteries by means of a catheter delivery system (*13, 22*). Initially, to determine cell types that could be transduced in vivo, arteries were infected with an adenovirus vector encoding a reporter gene, human placental alkaline phosphatase (*23*), immediately after balloon injury. Infection with this vector resulted in gene expression in smooth muscle cells in the intima and lumenal region of the media, as assessed by histochemical studies in arterial sections (Fig. 2, B to D). These data suggested that genetic modification of relevant vascular cell types could be achieved in vivo and used to modulate the response to injury. Although gene expression in such models might be transient, expression of the appropriate gene at the time of the injury might exert long-term effects on cellular proliferation and alter the diameter of the arterial lumen.

We next determined whether an ADV-tk vector could alter the development of intimal hyperplasia after balloon injury in vivo. The right and left iliofemoral arteries of domestic pigs were subjected to balloon injury for either 1 or 5 min, and the ADV-tk vector was immediately introduced into the arteries by catheter (*23*). Thirty-six hours after injury and artery transfection, a control group, transfected with the ADV-tk vector, received intravenous saline treatments (ADV-tk/−GC) (Fig. 3, A, B, and D). A separate group was administered a 6-day treatment of ganciclovir, 50 mg per kilogram of body weight per day (ADV-tk/+GC) (Fig. 3, A, B, and D). In additional studies, a control E1-deleted vector, ADV-ΔE1, was introduced into injured iliofemoral arteries, and animals were treated with ganciclovir (ADV-ΔE1/+GC) or saline (ADV-ΔE1/−GC) (Fig. 3, C and D). Three weeks after balloon injury and adenoviral infection, we measured the areas of the intima and the media in each artery by quantitative morphometry and determined the intimal to medial (I/M) area ratios. A significant reduction in the I/M area ratio of ~87% in the 1-min injury and ~54 to 59% in the 5-min injury was observed [as measured by *t* test or analysis of variance (ANOVA) with Dunnett's *t* test, respectively (both $P < 0.05$) (*23, 24*)] in animals transduced with ADV-tk and treated with ganciclovir compared with ADV-tk– or ADV-ΔE1–transduced animals treated with saline (Fig. 3). A reduction in intimal BrdC incorporation of 40% was observed in ADV-tk/+GC–treated animals compared with ADV-tk/−GC–treated animals 7 days after gene transfer (*25*), indicating that inhibition of smooth muscle cell proliferation contributed to this effect. No response was noted in animals receiving ADV-ΔE1 after treatment with ganciclovir (Fig. 3, C and D). The percent reduction of intimal hyperplasia in animals treated with ADV-tk/+GC was 59, 57, and 54% compared with animals treated with ADV-tk/−GC, ADV-ΔE1/−GC, and ADV-ΔE1/+GC, respectively. A significant reduction was observed 6 weeks posttreatment (Fig. 3D), suggesting that this reduction in intimal hyperplasia was stable. Such a reduction has not been observed after the introduction of other recombinant genes in the porcine model (*14*) and was of sufficient magnitude to potentially affect arterial blood flow.

Studies with adenoviral vectors administered through an endobronchial pulmonary route have raised questions about the immunogenicity and toxicity of this vector delivery system which might limit their utility in clinical settings (*26*). In our study,

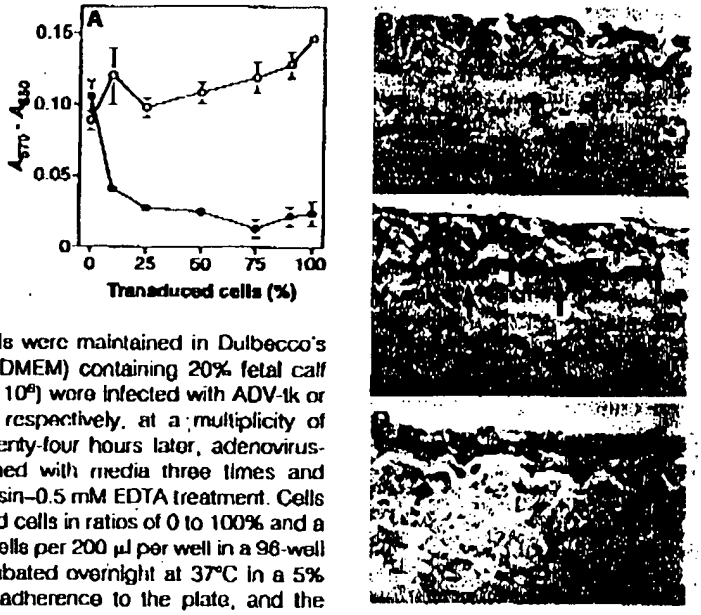

Fig. 2. In vitro sensitivity of porcine vascular smooth muscle cells to ganciclovir after ADV-tk infection, and transduction of porcine arteries by adenoviral vectors after catheter balloon injury in vivo. (**A**) Sensitivity of ADV-tk–transduced porcine vascular smooth muscle cells to ganciclovir. Primary pig aortic smooth muscle cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 20% fetal calf serum. Primary cells (1×10^6) were infected with ADV-tk or ADV-ΔE1 (10^{10} PFU/ml), respectively, at a multiplicity of infection of 300 (*35*). Twenty-four hours later, adenovirus-infected cells were washed with media three times and harvested with 0.05% trypsin–0.5 mM EDTA treatment. Cells were mixed with uninfected cells in ratios of 0 to 100% and a concentration of 1×10^4 cells per 200 μl per well in a 96-well plate. The cells were incubated overnight at 37°C in a 5% CO_2 incubator to permit adherence to the plate, and the media was then changed to fresh media containing 5 μM ganciclovir. Cultures were terminated at 5 days, and cell proliferation was measured by a colorimetric assay (*36*). Cell proliferation was proportional to the absorbance (**A**) at the test wavelength (570 nm) with subtraction of the reference wavelength (650 nm). Symbols: ●, cells infected with ADV-tk/+GC; ○, cells infected with ADV-ΔE1/+GC. Infection of injured arterial vascular smooth muscle cells with ADV-ΔE1 vector (**B**) or an adenoviral vector encoding human placental alkaline phosphatase (**C**) (*23, 35*). Histochemical staining for alkaline phosphatase was performed 5 days after injury and arterial transfection as described (*23*). Arrows denote positively stained, transduced cells. Immunohistochemical staining with an antibody to smooth muscle α-actin confirmed that transfected cells represented smooth muscle cells (**D**). Magnification ×160.

Table 1. Summary of organ pathology and serum biochemical parameters from pigs that received ADV-tk and ADV-ΔE1 vectors by catheter after balloon injury. Organs were analyzed from animals that received ADV-tk/−GC ($n = 8$), ADV-tk/+GC ($n = 8$), ADV-ΔE1/+GC ($n = 4$), and ADV-ΔE1/−GC ($n = 4$) treatment. CPK, creatine phosphokinase; LDH, lactate dehydrogenase; SGPT, serum glutamine pyruvate transferase; SGOT, serum glutamine oxaloacetic transferase; AP, alkaline phosphatase; BUN, blood urea nitrogen; CR, creatinine; N/A, no applicable tests.

Organ	Pathology	Serum biochemical parameters
Transfected artery	Adventitial mononuclear infiltrate	N/A
Nontransfected artery	Normal	N/A
Heart	Normal	CPK, LDH-normal
Lung	Normal	N/A
Liver	Normal	SGPT, SGOT, AP, bilirubin-normal
Kidney	Normal	BUN, Cr-normal
Spleen	Normal	N/A
Skeletal muscle	Normal	CPK
Ovary	Normal	N/A

the route of vector administration is intravascular, however, and the duration of expression is limited by expression of a suicide gene. Therefore, we examined the toxicity of this adenoviral vector delivered by arterial catheterization. Although the presence of vector could be detected by polymerase chain reaction (PCR) in major organs 5 days after gene transfer, expression of an alkaline phosphatase reporter gene could not be detected (*25*), suggesting very low levels of transduction. Analysis of artery tissue sections revealed no major adverse responses to the ADV-tk vector in vivo. In arteries, occasional mononuclear infiltrates were found in the adventitia with no evidence of vasculitis or necrosis in the intima. Tissues from the major organs of these animals, including nontransfected artery, heart, lung, liver, kidney, spleen, skeletal muscle, and ovary showed no significant pathological lesions, and standard serum biochemical parameters were within the normal range (Table 1). Thus, the in vivo toxicities of this adenoviral vector administered through an intra-arterial route were minimal.

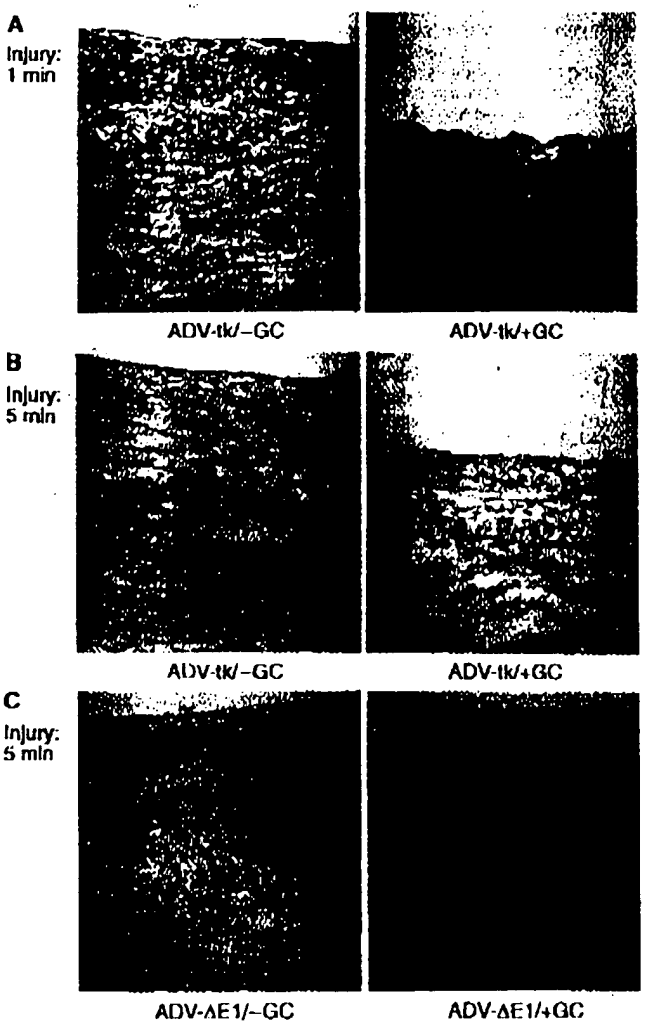

Fig. 3. Effect of ganciclovir on intimal and medial areas in arteries after balloon injury and infection with ADV-tk vector. Representative cross sections from iliofemoral arteries of pigs (**A**) injured for 1 min, then infected with ADV-tk vector and treated with saline (left) or ganciclovir (right); (**B**) injured for 5 min, then infected with ADV-tk vector and treated with saline (left) or ganciclovir (right); and (**C**) injured for 5 min, then infected with ADV-ΔE1 and treated with saline (left) or ganciclovir (right). These arteries were examined 3 weeks after injury and gene transfer (hematoxylin and eosin stain, magnification ×87). Measurements of I/M area ratios (**D**) are from arteries infected after a 1-min injury and 3 weeks posttransfection with ADV-tk vector and treated with saline (0.445 ± 0.047, $n = 4$ arteries) or ganciclovir (0.057 ± 0.027, $n = 4$ arteries); infected after a 5-min injury and 3 weeks posttransfection with ADV-tk vector and treated with saline (0.512 ± 0.047, $n = 8$ arteries) or ganciclovir (0.205 ± 0.065, $n = 8$ arteries); infected after a 5-min injury and 6 weeks posttransfection with ADV-tk vector and treated with saline (0.639 ± 0.099, $n = 4$ arteries) or ganciclovir (0.334 ± 0.024, $n = 4$ arteries); and infected after a 5-min injury and 3 weeks posttransfection with ADV-ΔE1 vector and treated with saline (0.481 ± 0.020, $n = 8$ arteries), or ganciclovir (0.445 ± 0.027, $n = 8$ arteries). A statistically significant reduction in I/M area ratios was observed in the ADV-tk/+GC group compared with ADV-tk/−GC (1 min, 3 weeks, two-tailed unpaired t test, $P < 0.05$); ADV-tk/+GC compared with ADV-tk/−GC, ADV-ΔE1/−GC, and ADV-ΔE1/+GC (5 min, 3 weeks, ANOVA with Dunnett's t test, $P < 0.05$); and ADV-tk/+GC compared with ADV-tk/−GC (5 min, 6 weeks, two-tailed unpaired t test, $P < 0.05$). I, intima; M, media; GC, ganciclovir.

Taken together, these data suggest that introduction of a replication-deficient adenoviral vector encoding herpesvirus tk limits intimal hyperplasia after balloon injury. This prodrug approach, in which high levels of recombinant *tk* gene are expressed locally, appears well suited to address the problem of vascular smooth muscle cell proliferation. This method provides for sustained expression and conversion of the prodrug to its toxic form locally at the time of peak cell proliferation at concentrations that cannot be achieved by drug delivery. Despite the success of several pharmacological (*27, 28*) and antisense oligonucleotide (*29*) interventions in balloon injuries in the rat, the applicability of the rat carotid artery injury model to human vascular disease is uncertain. For example, although angiotensin-converting enzyme inhibitors limited intimal hyperplasia in a rat carotid artery injury model (*28*), two larger international clinical trials subsequently showed a lack of effectiveness of these treatments in human restenosis (*3, 30*). Although the efficacy of ADV-tk gene transfer for balloon injury in this porcine model may provide a more relevant test than the injured rat carotid artery for its appropriateness in humans, the model still differs from human restenosis in that these pigs are not hyperlipidemic. It is known that gene transfer can be achieved in atherosclerotic rabbit arteries (*31*); however, it remains to be determined whether this approach will prove therapeutic for humans.

In theory, it would be advantageous to target vascular smooth muscle cells specifically in vivo, allowing the endothelium to resurface the arterial lumen which would reduce thrombogenicity (*32*) and vasoconstriction (*33*). Although ganciclovir is not selective for smooth muscle cells, it would appear that selectivity for dividing cells has been achieved in this study. The endothelial surface was found to regenerate at 3 to 6 weeks, as evidenced by morphology (Fig. 3, A to C), by scanning electron microscopy, and by vWF immunohistochemistry (*34*) in the injured arterial segment (*25*). The time course of endothelial cell regrowth was thus delayed, suggesting that reendothelialization occurred after ganciclovir treatment. In porcine iliofemoral balloon injury, reendothelialization may occur asynchronously with vascular smooth muscle growth (*20*). The ability to regenerate a significant amount of endothelium and to maintain patency of the lumen could compensate for the loss of endothelium in the short term while the capacity for reendothelialization at longer periods of time remains.

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35. The recombinant adenoviral vector, ADV-tk, was constructed by homologous recombination between sub360 genomic DNA, an Ad5 derivative with a deletion in the E3 region, and an HSV-tk expression plasmid, pAd-HSV-tk. These recombinant adenoviral vectors have sequences in the E1A and E1B region deleted, impairing the ability of this virus to replicate and transform nonpermissive cells. Briefly, the pAd-HSV-tk plasmid was prepared by introducing the Xho I–Hind III fragment of an HSV-tk expression vector, pIC 19R/MC1-TK [S. L. Mansour, K. R. Thomas, M. R. Capecchi, *Nature* 336, 348 (1988)], into the Bgl II site of pAd-Bgl II (*37*) which had the left-hand sequence of the Ad5 genome, but not E1A and E1B. A human embryonic kidney cell line that expresses E1, 293, was cotransfected with 10 μg of Nhe I–digested pAd-HSV-tk and 1 μg of Cla I and Xba I–digested sub360 DNA. Infectious viruses were isolated by plaque isolation, and plaques containing recombinant adenovirus were screened for tk activity. The E1A- and E1B-deleted adenovirus, ADV-ΔE1, was constructed by using the Eco RI–digested pAd-Bgl II plasmid with no insert in place of pAd-HSV-tk. The adenoviral vector encoding human placental alkaline phosphatase (ADV-hpAP) was constructed by insertion of a fragment from a hpAP expression vector under control of the cytomegalovirus enhancer into pAd-Bgl II, and the recombinant adenovirus was generated by homologous recombination between plasmid Ad-hpAP and Ad5 genomic DNA. The structure of these viruses was confirmed by Southern (DNA) blotting. All recombinant viruses were propagated in 293 cells and purified as described (*37*). Cesium chloride–purified virus was dialyzed against phosphate-buffered saline (PBS) and diluted for storage in 13% glycerol–PBS solution to yield a final concentration of 1×10^{12} to 3×10^{12} viral particles per milliliter (0.8×10^{10} to 5×10^{10} PFU/ml). All stocks were sterilized with a 0.45-μm filter and evaluated for the presence of replication-competent adenovirus by infection at a multiplicity of infection of 10 onto 3T3 cells. None of the stocks used in these experiments yielded replication-competent virus.
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